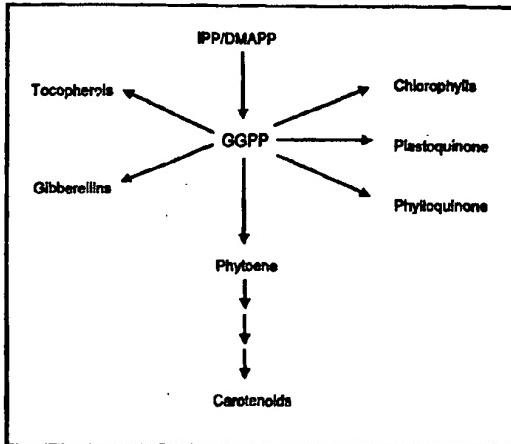




## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<p>(21) International Application Number: <b>PCT/EP00/01850</b></p> <p>(22) International Filing Date: 3 March 2000 (03.03.00)</p> <p>(30) Priority Data: 199 09 637.6 5 March 1999 (05.03.99) DE</p> <p>(71) Applicant (for all designated States except US): GREENOVATION PFLANZENBIOTECHNOLOGIE GMBH [DE/DE]; Sonnenstrasse 5, D-79104 Freiburg im Breisgau (DE).</p> <p>(72) Inventors; and</p> <p>(73) Inventors/Applicants (for US only): BEYER, Peter [DE/DE]; In der Etzmatt 10, D-79423 Heitersheim (DE). POTRYKUS, Ingo [DE/CH]; Im Stigler 54, CH-4312 Magden (CH).</p> <p>(74) Agent: JOACHIM STÜRKEN PATENTANWALTSGESELLSCHAFT MBH; Engesserstrasse 4b, D-79108 Freiburg im Breisgau (DE).</p>		
<p>(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).</p> <p>Published With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</p>		

(54) Title: METHOD FOR IMPROVING THE AGRONOMIC AND NUTRITIONAL VALUE OF PLANTS



## (57) Abstract

The present invention provides means and methods of transforming plant cells, seeds, tissues or whole plants in order to yield transformants capable of expressing all enzymes of the carotenoid biosynthesis pathway that are essential for the targeted host plant to accumulate carotenes and/or xanthophylls of interest. The present invention also provides DNA molecules designed to be suitable for carrying out the method of the invention, and plasmids or vector systems comprising said molecules. Furthermore, the present invention provides transgenic plant cells, seeds, tissues and whole plants that display an improved nutritional quality and contain such DNA molecules and/or that have been generated by use of the methods of the present invention.

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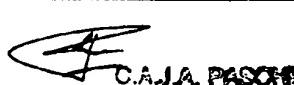
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Claims:

34

1. An isolated DNA molecule comprising a nucleotide sequence providing one or more expression cassettes capable of directing production of one or more enzymes specific for the carotenoid biosynthesis pathway selected from the group consisting of:
  - 5 - phytoene synthase derived from plants, fungi or bacteria,
  - phytoene desaturase derived from plants, fungi or bacteria,
  - $\zeta$ -carotene desaturase derived from plants, and
  - lycopene cyclase derived from plants, fungi or bacteria,under the proviso that an expression cassette capable of directing production of phytoene synthase alone is excluded.
- 10
2. The DNA molecule according to claim 1, wherein said expression cassette comprises one or more genes or cDNAs coding for plant, fungi or bacterial phytoene synthase, plant, fungi or bacterial phytoene desaturase, plant  $\zeta$ -carotene desaturase, or plant, fungi or bacterial lycopene cyclase, each operably linked to a suitable constitutive, inducible or tissue-specific promoter allowing its expression in plant cells, seeds, tissues or whole plants, under the proviso that an expression cassette comprising a gene or cDNA coding for phytoene synthase alone is excluded.
- 15
- 20
3. The DNA molecule according to claim 1 or 2, further comprising at least one selectable marker gene or cDNA operably linked to a constitutive, inducible or tissue-specific promoter sequence allowing its expression in plant cells, seeds, tissues or whole plants.
- 25
4. The DNA molecule according to any of claims 1 to 3, wherein the nucleotide sequence coding for phytoene synthase originates from plants, preferably expressed under the control of a tissue-specific promoter.
- 30
5. The DNA molecule according to any of claims 1 to 4, wherein the nucleotide sequence coding for phytoene desaturase originates from bacteria and is fused with a suitable plastid transit peptide encoding sequence, both of which preferably are expressed under the control of a tissue-specific or constitutive promoter.

6. The DNA molecule according to any of claims 1 to 5, wherein the nucleotide sequence coding for lycopene cyclase originates from plants, preferably expressed under the control of a tissue-specific or constitutive promoter.
- 5 7. The DNA molecule according to any of claims 2 to 6, wherein the selectable marker gene or cDNA is hygromycin phosphotransferase under the control of a constitutive promoter.
- 10 8. The DNA molecule according to any of claims 1 to 7, wherein said nucleotide sequence comprises functional expression cassettes for both phytoene synthase and bacterial or fungi phytoene desaturase.
9. The DNA molecule according to any of claims 1 to 7, wherein said nucleotide sequence comprises a functional expression cassette for lycopene cyclase.
- 15 10. The DNA molecule according to claim 5 or 8, wherein said plastid transit peptide sequence is derived from the pea Rubisco small subunit (tp).
11. A plasmid or vector system comprising one or more DNA molecules according to any of claims 1 to 10.
- 20 12. A plasmid or vector system according to claim 11, which is derived from *Agrobacterium tumefaciens*.
13. A transgenic plant cell, seed, tissue or whole plant that contains a DNA molecule according to any of claims 1 to 10.
- 25 14. A transgenic plant cell, seed, tissue or whole plant according to claim 13, selected from the group consisting of eukaryotic alga, embryophytes comprising *Bryophyta*, *Pteridophyta* and Spermatophyta such as *Gymnospermae* and *Angiospermae*, the latter including *Magnoliopsida*, *Rosopsida*, and *Liliopsida* ("monocots").
- 30

15. A transgenic plant cell, seed, tissue or whole plant according to claim 14, selected from the group consisting of grain seeds, with rice, wheat, barley, oats, amaranth, flax, triticale, rye, and corn being preferred; oil seeds, with *Brassica* seeds, cotton seeds, soybean, safflower, sunflower, coconut, and palm being preferred; other edible seeds or seeds with edible parts selected from the group consisting of pumpkin, squash, sesame, poppy, grape, mung beans, peanut, peas, beans, radish, alfalfa, cocoa, coffee, hemp; tree nuts, with walnuts, almonds, pecans, and chick-peas being preferred; potatoes, carrots, sweet potatoes, tomato, pepper, cassava, willows, oaks, elm, maples, apples, bananas; and ornamental flowers, with lilies, orchids, sedges, roses, buttercups, petunias, phlox, violets, and sunflowers being preferred.  
5  
10
16. A method of transforming plant cells, seeds, tissues or whole plants in order to yield transformants capable of expressing all enzymes of the carotenoid biosynthesis pathway necessary to produce carotenes and xanthophylls of interest, comprising the transformation of said plant cells, seeds, tissues or whole plants with one or more DNA molecules according to any of claims 1 to 10, or with a plasmid or vector system according to claim 11 or 12.  
15
17. A method according to claim 16, wherein said host plant cells, seeds or tissues selected for transformation normally are carotenoid-free.  
20
18. A method according to claim 16, wherein said host plant cells, seeds or tissues selected for transformation normally contain carotenoids in amounts desired to enlarge or modify.
- 25 19. A transformed whole plant regenerated from transformants yielded according to any of claims 16 to 18, or parts thereof, selected from the group consisting of eukaryotic alga, embryophytes comprising *Bryophyta*, *Pteridophyta* and Spermatophyta such as *Gymnospermae* and *Angiospermae*, the latter including *Magnoliopsida*, *Rosopsida*, and *Liliopsida* ("monocots").

20. A transformed whole plant or part thereof according to claim 19, selected from the group consisting of grain seeds, with rice, wheat, barley, oats, amaranth, flax, triticale, rye, and corn being preferred; oil seeds, with *Brassica* seeds, cotton seeds, soybean, safflower, sunflower, coconut, and palm being preferred; other edible seeds or seeds with edible parts selected from the group consisting of pumpkin, squash, sesame, poppy, grape, mung beans, peanut, peas, beans, radish, alfalfa, cocoa, coffee, hemp; tree nuts, with walnuts, almonds, pecans, and chick-peas being preferred; potatoes, carrots, sweet potatoes, tomato, pepper, cassava, willows, oaks, elm, maples, apples, bananas; and ornamental flowers, with lilies, orchids, sedges, roses, buttercups, petunias, phlox, violets, and sunflowers being preferred.

**INTERNATIONAL SEARCH REPORT**

Inte	Application No
PCT/EP/01850	

<b>A. CLASSIFICATION OF SUBJECT MATTER</b>					
IPC 7	C12N15/52	C12N15/53	C12N15/82	C12N5/10	A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, CHEM ABS Data

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 872 554 A (HOFFMANN LA ROCHE) 21 October 1998 (1998-10-21) the whole document -----	1,11
X	BARTLEY GLENN E ET AL: "Two Arabidopsis thaliana carotene desaturases, phytoene desaturase and zeta-carotene desaturase, expressed in Escherichia coli, catalyze a poly-cis pathway to yield pro-lycopene." EUROPEAN JOURNAL OF BIOCHEMISTRY, vol. 259, no. 1-2, January 1999 (1999-01), pages 396-403, XP000925505 ISSN: 0014-2956 the whole document ----- -/-	1,11

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

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## INTERNATIONAL SEARCH REPORT

Int'l Application No  
PCT/EP/01850

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 36717 A (CENTRE NAT RECH SCIENT ;KUNTZ MARCEL (GB)) 21 November 1996 (1996-11-21) the whole document	1-3, 6, 7, 9, 11, 12
X	US 5 539 093 A (FITZMAURICE WAYNE P ET AL) 23 July 1996 (1996-07-23)  the whole document	1-3, 7, 11-16, 18-20
X	MISAWA N ET AL: "EXPRESSION OF AN ERWINIA PHYTOENE DESATURASE GENE NOT ONLY CONFERS MULTIPLE RESISTANCE TO HERBICIDES INTERFERING WITH CAROTENOID BIOSYNTHESIS BUT ALSO ALTERS XANTHOPHYLL METABOLISM IN TRANSGENIC PLANTS" PLANT JOURNAL, GB, BLACKWELL SCIENTIFIC PUBLICATIONS, OXFORD, vol. 6, no. 4, 1994, pages 481-489, XP002012919 ISSN: 0960-7412 the whole document	1-3, 7, 10-16, 18-20
X	WO 98 06862 A (SHEWMAKER CHRISTINE K ;CALGENE INC (US)) 19 February 1998 (1998-02-19) cited in the application see the whole document; esp. examples	1-20
X	WO 99 07867 A (CALGENE LLC) 18 February 1999 (1999-02-18) see the whole document; esp. examples	1-20
X	WO 91 13078 A (AMOCO CORP) 5 September 1991 (1991-09-05) cited in the application see the whole document; esp. ex.18	1-3, 5, 7-20
A	BURKHARDT ET AL: "Transgenic rice ( <i>Oryza sativa</i> ) endosperm expressing daffodil ( <i>Narcissus pseudonarcissus</i> ) phytoene synthase accumulates phytoene, a key intermediate of provitamin A biosynthesis" PLANT JOURNAL, GB, BLACKWELL SCIENTIFIC PUBLICATIONS, OXFORD, vol. 11, no. 5, 1997, pages 1071-1078, XP002087151 ISSN: 0960-7412 cited in the application see the whole document; esp. last paragraph	1-20
	-/-	

## INTERNATIONAL SEARCH REPORT

Inte [REDACTED] Application No  
PCT/EP 00/01850

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 96 13149 A (AMOCO CORP) 9 May 1996 (1996-05-09) cited in the application the whole document	1-20
A	US 5 705 624 A (KUMAGAI MONTO HIROSHI ET AL) 6 January 1998 (1998-01-06) the whole document	1-20
A	AL-BABILI S. ET AL.: "Narcissus lycopene cyclase cDNA; AC X98796" EBI DATABASE, 4 July 1996 (1996-07-04), XP002143214 the whole document	1,2,6,9
P,X	YE, XUDONG ET AL: "Engineering the provitamin A (.beta.- carotene ) biosynthetic pathway into ( carotenoid -free) rice endosperm" SCIENCE (WASHINGTON, D. C.) (2000), 287(5451), 303-305 , XP002143215 the whole document	1-20

## INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP 00/01850

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
EP 0872554	A	21-10-1998	BR 9705676 A CN 1184159 A JP 10155497 A		25-05-1999 10-06-1998 16-06-1998
WO 9636717	A	21-11-1996	AU 5897796 A		29-11-1996
US 5539093	A	23-07-1996	NONE		
WO 9806862	A	19-02-1998	AU 4058497 A CN 1227609 A EP 0925366 A		06-03-1998 01-09-1999 30-06-1999
WO 9907867	A	18-02-1999	AU 8900298 A EP 1002117 A		01-03-1999 24-05-2000
WO 9113078	A	05-09-1991	CA 2055447 A EP 0471056 A JP 5504686 T US 5545816 A US 5530188 A US 5530189 A US 5684238 A US 5618988 A US 5656472 A		03-09-1991 19-02-1992 22-07-1993 13-08-1996 25-06-1996 25-06-1996 04-11-1997 08-04-1997 12-08-1997
WO 9613149	A	09-05-1996	US 5618988 A AU 697358 B AU 3970195 A CA 2203815 A CN 1172416 A EP 0792352 A JP 10509309 T NO 971945 A NZ 296012 A PL 319788 A		08-04-1997 01-10-1998 23-05-1996 09-05-1996 04-02-1998 03-09-1997 14-09-1998 27-06-1997 28-05-1999 01-09-1997
US 5705624	A	06-01-1998	NONE		